Week 5

July 5, 2016

What We Did Today:

Checked competency of cells using iGEM 2016 cell competency kit and DH5(alpha) cells

Transformed neg control, 0.5, 5, 10, 20, 50

Made Liquid LB

Made 2x AMP plates

Liquid culture of 12 colonies from the ligation transformation from Thursday 2x amp (10uL amp)
The plates looked good
about 7 colonies neg control, double on 2uL insert and 3-4 on 7uL and 15uL insert.

Tested Competency of new DH5Alpha cells
1uL of tester DNA in 50 uL cells and 1 control on CAM plates
Digested Purified APOBEC1 (1xXten, 2xXten, 3xXten) with PacI and NotI, incubated for 2 hours

• Liquid culture:
EMG2Kλ transformed with dCas9-pRetro ligation, 2X Amp LB (10 μL/tube), 12 samples
• 2X Amp LB plates

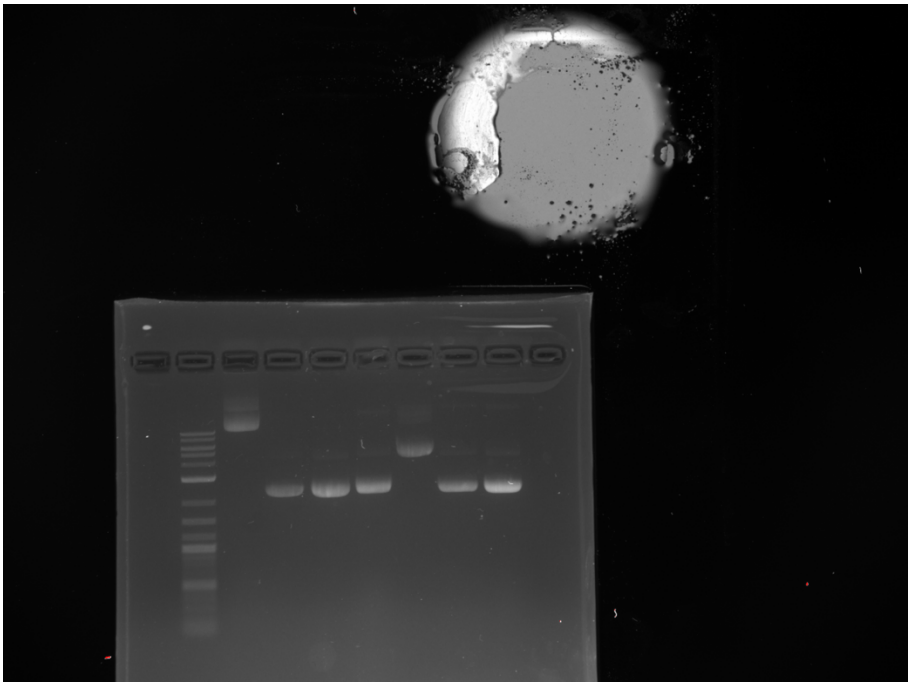
July 6, 2016

Plates from Cell Competency Test:
- Grow on all plates except negative control.
- Plates with more DNA had more growth
- Successful transformation efficiency

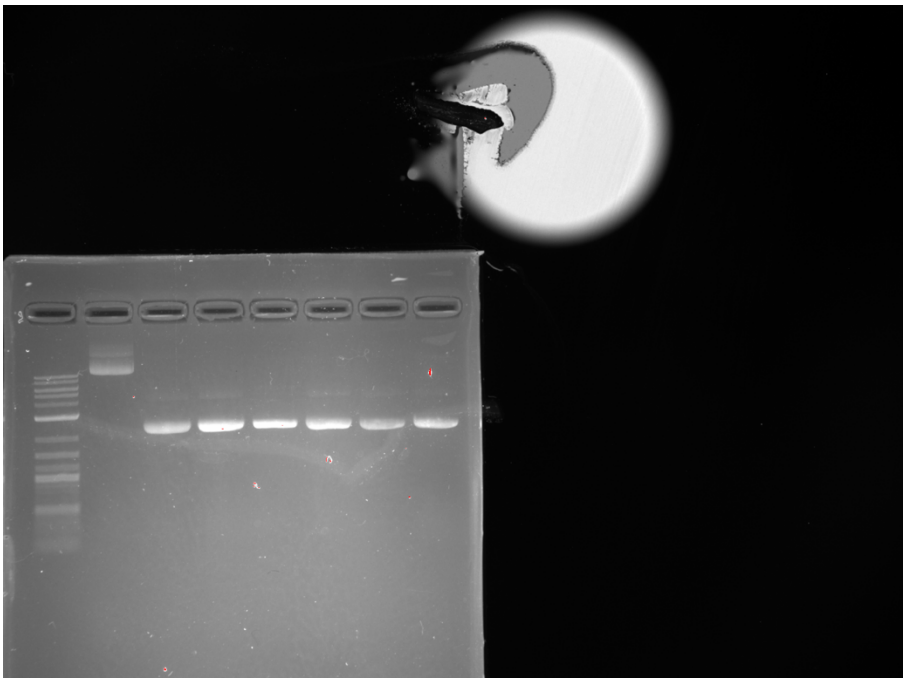
Mini-Prep pRETRO-ON-dCas9 clones (12 total)
- Clone 1 – 229.9 ng/uL
- Clone 2 – 344.6 ng/uL
- Clone 6 – 334.4 ng/uL
- Clone 9 – 129.4 ng/uL
- Clone 10 – 228.2 ng/uL
- Clone 12 – 187.1 ng/uL

Digested mini-preps using protocol:
- 1ug (volume to be determined) plasmid
- 2uL cutsmart buffer
- 1uL BamH1
- 1uL Not1
- Adjust volume to 20uL

Ran undigested pRETRO-On-dCas9 on gel



Gel 1:
Lane 1 – Empty
Lane 2 - Ladder
Lane 3 – Negative Control
Lane 4 – Sample 1
Lane 5 – Sample 2
Lane 6 – Sample 3
Lane 7 – Sample 4
Lane 8 – Sample 5
Lane 9 – Sample 6



Gel 2:
Lane 1 – Ladder
Lane 2 - Negative Control
Lane 3 – Sample 7
Lane 4 – Sample 8
Lane 5 – Sample 9
Lane 6 – Sample 10
Lane 7 – Sample 11
Lane 8 – Sample 12

Analysis:

All lanes should have been around 6.8kb. The negative control was high because it was uncut and supercoiled. The other samples seem to have lost about 4kb and the reason is unknown. Sample 3 is not the same as the others but is still missing DNA when compared to the control. It is thought that this is because of the use of EMG2k(lambda) cells for transformation.

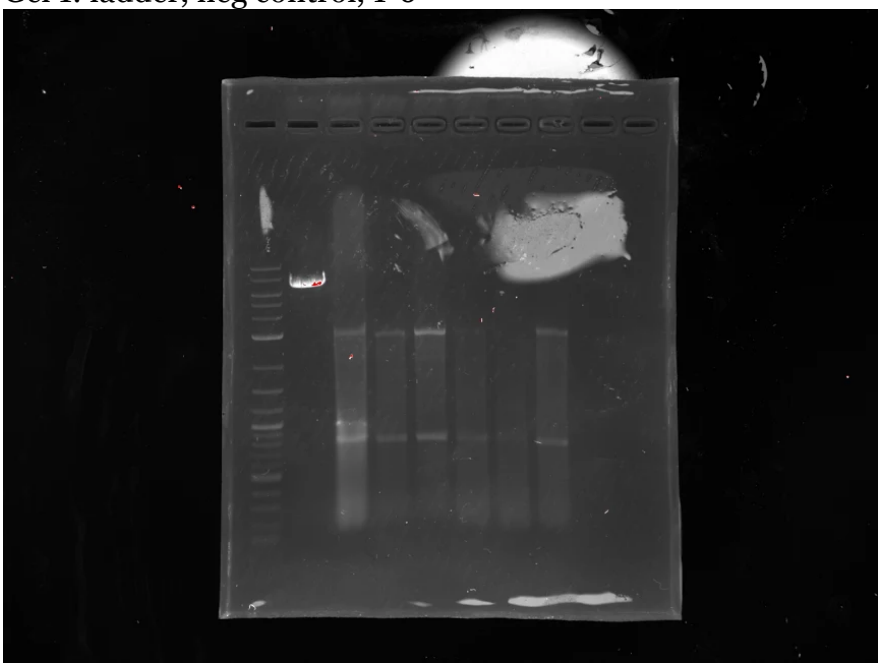
Mini prepped ligation liquid cultures
#5 – 220.2 ng/uL
#11 – 214.4 ng/uL

1ug #5 = 4.54uL (11.46uL water)
1ug # 11 = 4.67uL (11.33uL water)

test digest with BamH1 and Pac1
little Pac1 left so we switched to Not1 and BamH1 because Not1 is really close to Pac1

run gel to test

Gel 1: ladder, neg control, 1-6



Gel 2: ladder, neg control, 7-12

Prepped new vector, started over

PCR ADAR 2 -2x insert

• Glycerol stocks for 12 cultures (pRetro ON-dCas9)
    - 250 µL culture
    - 250 µL glycerol

- Miniprep
  - 7: 312.0 ng
  - 8: 223.3 ng
- Digest
  - DNA: pRetro-dCas9
  - Enzymes: BamHI, NotI
    - 1 µg DNA (7: 3.2 µL; 8: 4.5 µL)
    - Total 20 µL
- Digest (vector prep)
  - DNA: pRetro
  - Enzymes: BamHI, PacI
    - 10 µL vector
    - 2 µL each enzyme
- PCR
  - ADAR1-2X linker
    - Cycle * 34

July 7, 2016

Prepared New Vectors

1.) Prepare Mixtures

PcDNA 3.1

616.9 ng/ul = 1.62 ug/ul
10 ug DNA = 16.2 ul
Mixture:

16.2 ul DNA
3 ul Cutsmart buffer
2 ul BamH1
2 ul xBa1
26.8 ul H20

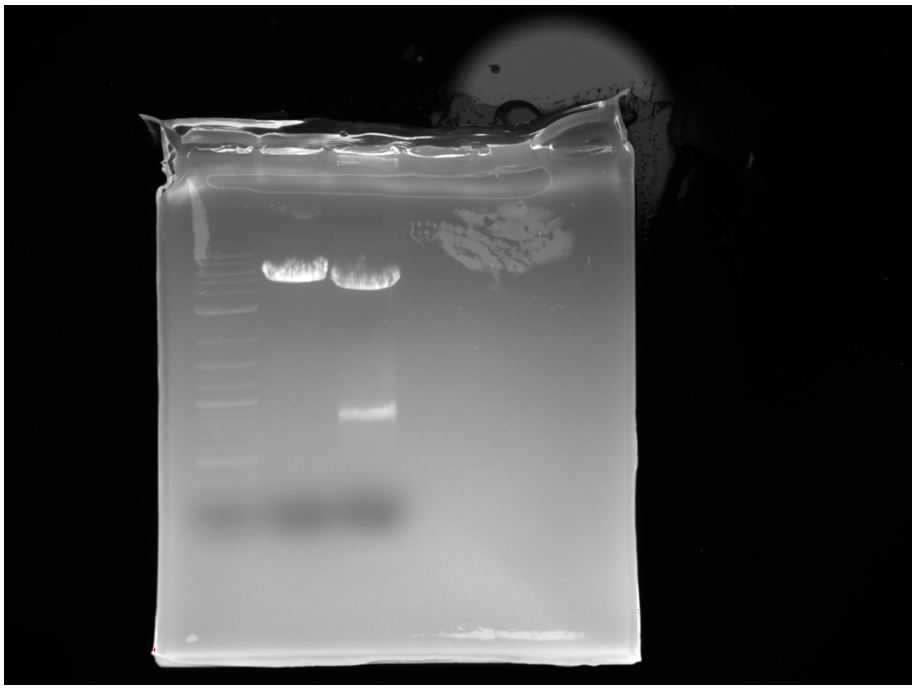
PEGFP N3

863.0 ng/ul = 1.16 ug/ul
10 ug = 11.59 ul
Mixture:

11.59 ul DNA
3 ul Cutsmart buffer
2 ul xH01
2 ul BamH1
31.41 ul H20

2.) Incubate in 37 degree waterbath for at least 2 hours
3.) Add 1 ul SAP
4.) Incubate in 37 degree for 20 mins
5.) Heat kill at 80 degrees
6.) Run total volume on gel (Spin down before adding loading dye)
(Dye = 1/5 pf volume)
Take pictures

Vector Gel



Lane 1 – Ladder
Lane 2 – pcDNA 3.1
Lane 3 – pEGFP N3

Gel Analysis:

The band for the pcDNA 3.1 is between the 5 and 6kb marks according to the ladder. This is correct because this plasmid is 5.4kb. The band for the pEGFP N3 is above the 3kb mark but below the 5kb mark according to the ladder. This is correct because this plasmid is 4.7kb.

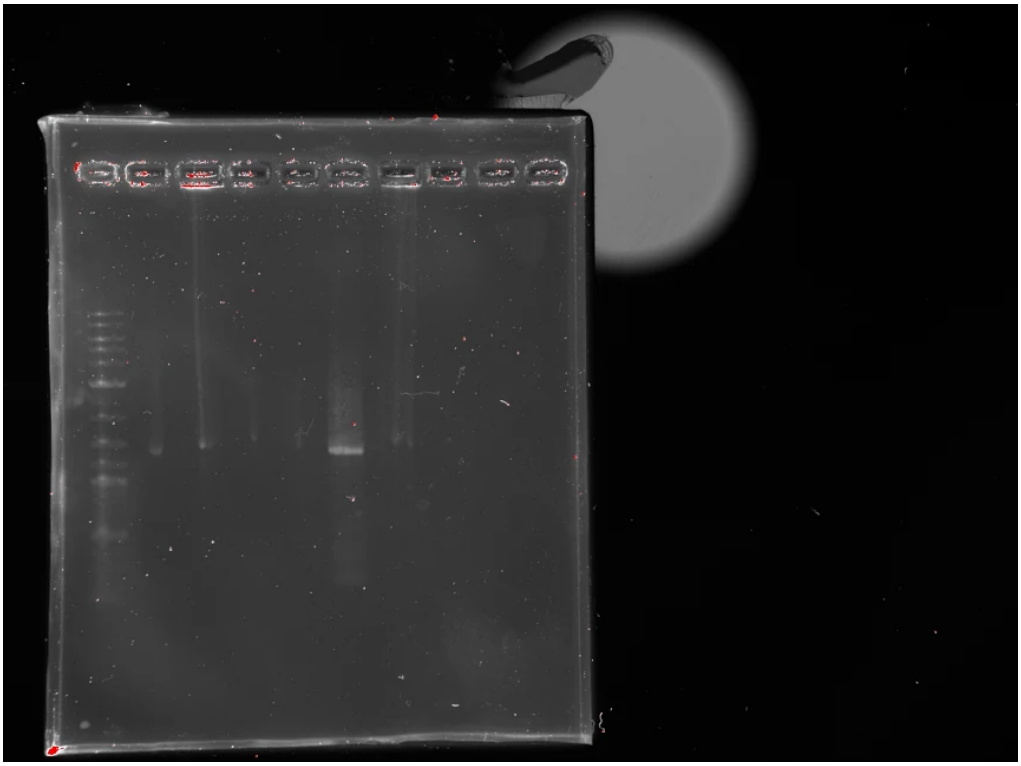
7.) Gel extraction
- Weights:
  - pcDNA 3.1 - 0.6g -> added 1.2ml Orange Gatorade
  - PEGFP N3 – 0.5g -> added 1ml Orange Gatorade
- Extraction column
  - 25ul Elution 1 – sit 2-3 mins
  - Spin
  - 25 ul Elution 2 – sit 2-3 mins
  - Spin

8.) Nanodrop to determine how much DNA was lost during process
- Final Readings:
  - PcDNA 3.1 - 61.7 ng/ul -> retained 60% of original DNA
  - PEGFP N3 – 112.3 ng/ul -> retained 50% of original DNA

SAP 1uL 37 degree water bath 20 mins (cont. old vector prep)

PCR cleanup ADAR

Gel ADAR:



Ladder, 1-1, 1-2, 1-3, 2-1, 2-2, 2-3

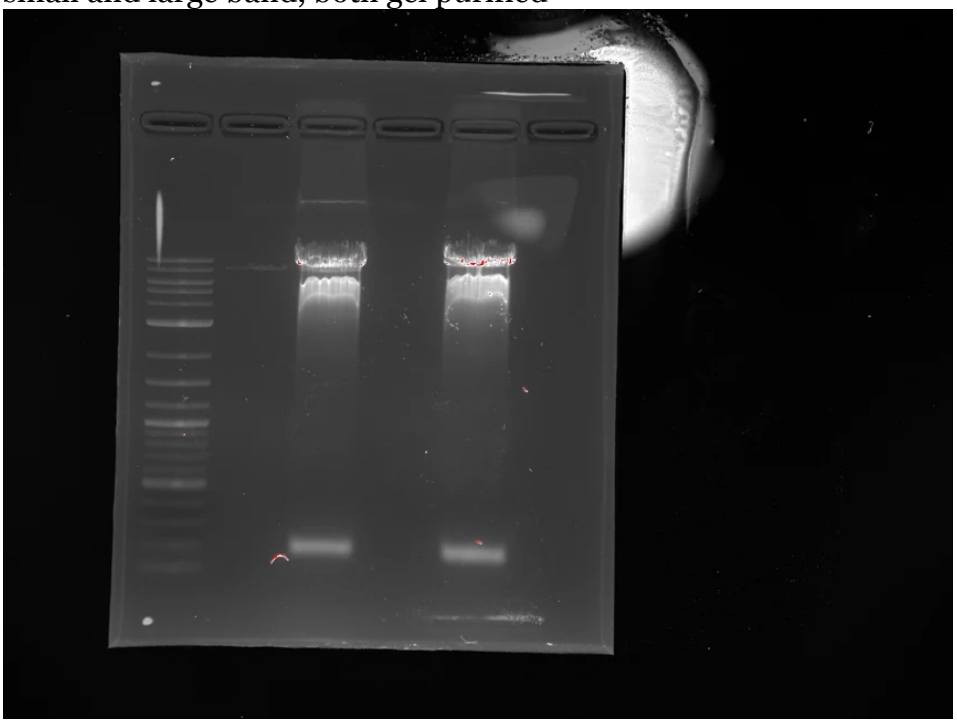
Digest ADAR :
43uL ADAR
6uL buffer
2uL each enzyme (Pac1, BamH1)
7uL water

No SYBR in loading dye fix:
2uL SYBR + 20mL 1xTAE
Place in box and swish 20 mins

Gelled vector, did gel purifications cut too much gel out (to fix did wash step 2x)

pgRNA prep

small and large band, both gel purified



dCas9 insert Cleanup, checking for presence of dCas9

- PCR clean up
  ADAR1-2X, ADAR1-3X
  - Double elution (25 µL + 25 µL)
- Gel
  ADARs * 6
  - 5 µL DNA
  - 1 µL dye

  Lanes (L to R): Ladder, ADAR1-1X, ADAR1-2X, ADAR1-3X, ADAR2-1X, ADAR2-2X, ADAR2-3X
- Digest
  ADARs * 6 (~45 µL each)
  Enzymes: PacI, NotI
  - 7 µL H_2O
  - 6 µL buffer
- Gel + gel purification
  pRetro (7/6/16 prep)
  - 60 µL total, 2 wells
  - 1.5 g gel + ~3 mL NTI
  - 58.3 ng/µL
- Digest (vector prep)
  DNA: pgRNA
  Enzymes: BstXI, XhoI
  - 3.1 buffer, 5 µL
  - 10 µg (12 µL) DNA (0.833 µg/µL)

- 2 µL each enzyme
- Total = 50 µL

• Gel + gel purification

pgRNA

- Top (small) band: 300 mg → 600 µL NTI → 70.1 ng
- Bottom (large) band: 600 mg → 1200 µL NTI → 23.2 ng

June 8, 2016

Ligation Reaction using pRETRO-ON vector and dCas9 insert (2-3/small insert)

Using old ligation protocol

1.) Made 4 Mixtures with 2 ul vector in each:
   1.) 0 ul insert and 15 ul water
   2.) 2 ul insert and 13 ul water
   3.) 8 ul insert and 7 ul water
   4.) 15 ul insert and 0 ul water
2.) Heat shocked in 80 degrees for 2 mins
3.) Removed heating block and let cool to room temp
4.) Added 2 ul buffer and 1ul T4 Ligase
5.) Incubated at room temp for 30 mins
6.) Transformed 5 ul and 10 ul using DH5(alpha) cells onto 2X Amp plates

Transformed pgRNA bands into DH5alpha cells

Ligation:
(New instant ligation mix, small dCas insert)
8uL insert/ 2uL insert and 6uL water/ or 8uL water
80 C 2 min take heat block out let it goe to room temp
10uL ligation mix pipette up and down
Transform (5 uL and 10 uL)into DH5alpha cells

• Transformation
   Cells: DH5α
   Small/large band pgRNA
• Ligation (with instant ligation mix)
   Vector: pRetro
   Insert: dCAS9
   - Water: 8, 2, 0 μL
   - Insert: 0, 6, 8 μL
   - 10 μL instant mix
   - 2 μL vector
   - Heat to 80 ˚C, let cool gradually
• Transformation
   Cells: DH5α

   - 5/10 μL ligation product